

**REPUBLIC OF TURKEY
ERCIYES UNIVERSITY
GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
DEPARTMENT OF AGRICULTURAL SCIENCES AND
TECHNOLOGIES**

**APPLICATION OF NANOPARTICLES AND OREGANO
(*Origanum vulgare*) ESSENTIAL OIL AGAINST GRAY
MOLD DISEASE AGENT (*Botrytis cinerea*)
ON TOMATO**

**Prepared By
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**Supervisor
Prof. Dr. Mehmet ARSLAN**

M. Sc. Thesis

**July 2018
KAYSERİ**

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SCIENTIFIC ETHICS SUITABILITY

#

I am, Salah Mohammed ABDULRAZAQ hereby declare that this piece of work is the result of my research and that this work has never been submitted anywhere for any degree. All the sources of information used were obtained in accordance with academic ethics and have been duly acknowledged.

Salah Mohammed ABDULRAZAQ



COMPLIANCE WITH GUIDE

This master's thesis write-up on the topic "Application of Nanoparticles and Oregano Essential Oil Against Gray Mold Disease Agent *Botrytis cinerea* On Tomato" was prepared with accordance Erciyes University's graduate Thesis Writing Directive

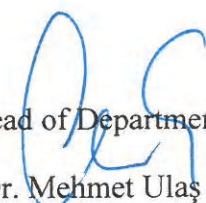
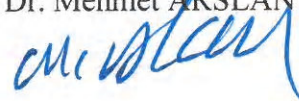
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ACCEPTANCE AND APPROVAL

This study entitled “Application of Nanoparticles and Oregano Essential Oil Against Gray Mold Disease Agent *Botrytis cinerea* on Tomato”, prepared by Salah Mohammed ABDULRAZAQ under the supervision of Prof. Dr. Mehmet ARSLAN, has been accepted by the jury as a thesis for the MSc at Erciyes University, Graduate School of Natural and Applied Sciences, Department of Agricultural Sciences Technologies.

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The acceptance of this thesis was based on decision number 31/07/2018 of the Executive Board of the Graduate School dated 2018/33-19

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Director of the Institute
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Kayseri July 2018

**APPLICATION OF NANOPARTICLES AND OREGANO (*Origanum vulgare*)
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AGENT (*Botrytis cinerea*) ON TOMATO**

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Erciyes University, Graduate School of Natural and Applied Science

M. Sc. Thesis, July 2018

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ABSTRACT

The antifungal activity of ZnO and CuO nanoparticles and their mixtures with oregano (*Origanum vulgare*) essential oil were tested *in vitro* and *in vivo* to control gray mold disease (*Botrytis cinerea*) of tomato. The 0, 5, 50, 100, and 200 mg/l concentrations of nano ZnO and CuO were used to determine their antifungal effect *in vitro* growth of *Botrytis cinerea*. 50 and 200 mg/l of ZnO and CuO nanomaterials alone and their essential oil mix with 10, 20, 40, 80, 160, 320 and 640 µl/l concentrations were tested on *B. cinerea* growth on tomato leaf disc *in vitro*. Also 0, 50 and 200 mg/l of nano ZnO and CuO were used alone and mixed with 80 and 160 µl/l oregano essential oil in an *in vivo* whole plant experiment to test their antifungal effect on *B. cinerea*. *In vitro* petri dish assays showed that both nanoparticles had significant inhibitory effect on the colony formation of *B. cinerea*. *In vivo* inoculation assays further confirmed that both CuO and ZnO nanoparticles significantly decreased colony formation of spores and disease growth on tomato. Antifungal activity of nano ZnO and CuO significantly increased when mixed with oregano essential oil. Antifungal effects of nanoparticles and essential oil increased as the concentrations of nanoparticles and essential oil increased. Oregano essential oil mixed nano ZnO or CuO could be used as possible fungicide against *B. cinerea* on tomato plant.

Key words: *Botrytis cinerea*, CuO, essential oil, *Origanum vulgare*, tomato nanoparticles, ZnO

DOMATESTE KURŞUNİ KÜF (*Botrytis cinerea*) HASTALIĞI ÜZERİNE NANOPARTİKÜL VE KEKİK (*Origanum vulgare*) UÇUCU YAĞININ ETKİSİ

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ÖZET

Domateste kurşuniküfhastalığı(*Botrytis cinerea*) üzerinde ZnO ve CuO nano partiküllerinin ve nanopartikülkekik (*Origanum vulgare*) uçucu yağı karışımlarının antifungal aktivitesi hem *in vitro* hende *in vivo* olarak test edilmiştir. Kurşuni küf gelişimi üzerine 5, 50, 100, and 200 mg/l konsantrasyonlardaki nano ZnO ve CuO'nun antifungal etkisi *in vitro* olarak tespit edilmiştir. Ayrıca *in vitro* koşullarda 50 ve 200 mg/l konsantrasyonda ZnO ve CuO 0, 10, 20, 40, 80, 160, 320 and 640 µl/l kekik uçucu yağı ile karıştırılarak yaprak diskleri üzerinde kurşuni küf gelişimi test edilmiştir. Sera koşullarında her iki nanomateryal 0, 50 ve 200 mg/l konsantrasyonlardan nano ZnO ve CuO 80 ve 160 µl/l uçucu yağla karıştırılıp tüm bitki üzerine uygulanarak *in vivo* antifungal etkileri belirlenmiştir. *In vitro* denemeleri her iki nanomateryalinde koloni oluşumu üzerinde baskılayıcı etkiye sahip olduğunu göstermiştir. *In vivo* inokülasyon denemeleri de CuO ve ZnO nano partiküllerinin domates üzerinde sporların koloni oluşumunu azalttığını ve hastalığın ilerlemesini engellediğini teyit etmiştir. Nano ZnO ve CuO partiküllerinin antifungal etkisi uçucu yağ ile birlikte karışım olarak kullanıldığında önemli derecede artmıştır. Nano partiküllerin ve uçucu yağın antifungal etkisi nano partikül konsantrasyonunun artışıyla birlikte artmıştır. Kekik uçucu yağı ile karıştırılan nano ZnO ve CuO domateste kurşuni küf hastalığına karşı muhtemel fungusit olarak kullanılabileceği belirlenmiştir.

Anahtar kelimeler: *Botrytis cinerea*, domates, CuO, *Origanum vulgare*, uçucu yağ, nanopartiküller, ZnO

CONTENTS

APPLICATION OF NANOPARTICLES AND OREGANO (*Origanum vulgare*) ESSENTIAL OIL AGAINST GRAY MOLD DISEASE AGENT (*Botrytis cinerea*) ON TOMATO

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CHAPTER 1

INTRODUCTION

Botrytis cinerea has gained great attention due to the increased global trade in cold-stored products since it can grow successfully for a long period just above the freezing temperatures in the cold-stored products such as cucumber, apples and pears. Likewise, vegetable export is adversely affected by *B. cinerea*. Pande *et al.* (2002) reported that out-of-season vegetable cultivations such as cucumber, tomato and sweet pepper under heated or unheated greenhouses and plastic tunnels had serious the treat of *B. cinerea* infection, especially.

The possibilities of control of *B. cinerea* are numerous. Continuous day and night ventilation in order to keep plants dry and to keep humidity low are essential to prevent and control *B. cinerea*. Girard *et al.* (2016) indicated that chemicals used to control *B. cinerea* were not effective, since proper environmental conditions favor the gray mold growth, development and spread.

Optimum hygienic conditions are very important to *B. cinerea* control. This is obtained by cutting out the stem infections and removing the dead plant before pruning operation beginning (Fiume and Fiume, 2006). To prevent the buildup of spores and the spread of infection, it is useful cutting out of the infected plant stems before the whole plant is infected. This method saves the uninfected plants from infection by removing all plant debris from the previous crop. Disposing plant residues away from the cultivated areas is a good method to stop *B. cinerea* survival in the soil and eliminate infection sources for the following crops (Girard *et al.*, 2016).

Chemical control of *Botrytis* gray mold is another strategy to control this phytopathogenic fungus. Therefore, synthetic chemicals alone cannot control the gray mold disease. The application of the chemicals is difficult to wash all surfaces of the

plants where infection may happen. The chemical fungicides presently registered are protective and they have no systemic action to control *Botrytis* gray mold. Heydari and Pessarakli (2010) reported that chemical fungicides simply provide a protective barrier to the surface of the plant that depresses fungal spore development. Chemical fungicides do not recover the diseased plants once the disease has developed on the healthy plants. Chemicals help reduce the risk of infection along with good ventilation and hygiene. Chemicals used on tomato plants in greenhouse are iprodione, procimidone, pyrimintanil, ciprodinil + fludioxonil. Kageyama and Nelson (2003) indicated that the chemical application would not be necessary to exceed 1 or 2 per a year. It is better to change the products and intervene the presence of initial hotbeds. *B. cinerea* has developed resistance to several chemicals (Moorman and Lease, 1992). However, there is an increased concern about selection for additional pathogen resistance to chemical fungicides in the near future. Tripathi and Dubey (2004) reported that many labeled chemical fungicides for *B. cinerea* on tomato were under inspection as possible carcinogens. Upcoming availability of chemicals for vegetables to control *B. cinerea* may be inadequate due to the environmental concerns and human health (Antunes and Cavaco, 2010). The occurrence of fungicide resistance and increasing consumer demands for decreased residues on vegetables and fruits highlights the need for alternative plant disease control measures. Another possibility of control of *B. cinerea* is represented by the use of biological means. Bio-control measures, as alternative strategies, have the advantages due to the higher public acceptance and lowered environmental contaminations. (Martinez-Romero *et al.*, 2008 ; Gachango *et al.*, 2012).

A new method to control of postharvest pathogens such as *B. cinerea*, while maintaining fruit quality, has been applied by the use of essential oils. This approach eliminates the need for artificial fungicides, thus conforming the consumer choices, ecologic requirements and lowering the environmental contaminations (du Plooy *et al.*, 2009). Camele *et al.* (2010) stated that thyme (*Thymus vulgaris*) essential oil stopped fruit rot caused by *B. cinerea*, *Phytophthora citrophthora*, and *Rhizopus stolonifer*; however, it was ineffective against *Penicillium italicum*. Furthermore, essential oils of *Origanum vulgare* and *Verbena officinalis* stopped the infection by *B. cinerea*, *Phytophthora citrophthora*, and *Rhizopus stolonifer*.

Currently, nanotechnology is a promising interdisciplinary research area and it has many useful applications in the agricultural industry. Nowadays, it receives great attention in practice due to the potential benefits of nanomaterials such as pest management. Traditional disease management strategies often prove inadequate measures, and application of synthetic chemical pesticides has negative effects on environment and human health. Nanotechnology can offer more safe and efficient alternatives for plant disease control, while the most promising strategies, in this scenario, are the use of nanoparticles to promote a more efficient assembly and then release of specific and environmental sustainable active principles. The wide range of nanotechnology applications in agriculture comprises nano-pesticides for the control of crop pests and diseases. Fungi, bacteria, insects, nematodes, phytoplasmas, and viruses cause plant diseases that increase economic losses by reducing attainable yields, product quality, shelf life; only in the United States, over 600 million dollars is spent yearly on fungicides in an effort to manage plant diseases (Combrinck *et al.*, 2011).

The use of natural compounds such as plant extracts can be an alternative option for synthetic chemicals to control fungal pathogens. A naturally occurring phenolic compound carvacrol is used as an antifungal agent. Carvacrol is a major component of oregano (*Origanum* sp) essential oil and it is existing in essential oil of many other plant species including *Thymbra spicata*, *Coridothymus* ssp. and *Satureja* sp.

Keeping all these points mentioned above an effort was made in the present work to evaluate the potential use nanoparticles alone and mixed with oregano essential oil to control *Botrytis cinerea* on tomato. Therefore, the objective of this study was to evaluate the possibilities of using oregano essential oil mixed with nanoparticles to control *Botrytis cinerea* on tomato.

CHAPTER 2

LITERATURE REVIEW

2.1. Tomato fruit

Tomato fruit (*Solanum lycopersicum*) is one of the most important vegetables in the world market because of its wide use and nutritious value. Tomato has a short shelf life due to postharvest diseases and transpiration. Tomato can be eaten fresh or can be fried, stewed, baked, or juiced. In addition to this versatility, tomatoes are also nutritional. Tomato species have low calories and have great iron and vitamin A and C contents. Fanasca *et al.* (2006) stated that tomatoes have high carote, such as lycopene with antioxidant properties, and lower quantities of the B complex vitamins thiamin, niacin, and riboflavin.

2.1.1. History

It was assumed that Andes Mountains in Peru and Ecuador is the origin of the wild potato species. Tomato is believed to have been domesticated in pre-Columbian Mexico since its name comes from the Náhuatl (Aztec) word *tomatl*. Spanish sailors introduced tomato to the Europe in the first decade of 16th century. It is assumed that Spanish and Italians are the first Europeans to use it as a food. Initially, tomato is grown as an ornamental plant in France and northern European countries since it was considered a suspicious food since botanists announced tomato due to the deadly nightshade (*Atropa belladonna*). Actually, the leaves and the roots of the tomato are toxic and comprise a neurotoxin solanine (Bergougnoux, 2014).

Europeans were introduced the tomatoes to North America. It was known that Thomas Jefferson grew tomato at Monticello in 1781. Tomato fruits were first used as food in Louisiana in the early as 18th century, but not in the northern states until 1835. Tomato

did not gain widespread attention in the United States of America until the early 20th century. Now tomato is commercially cultivated, and it is the second most consumed vegetable in the world behind only the potato throughout the world.

2.1.2. Tomato Anatomy

The tomato is usually classified as a vegetable, but it is actually a fruit. Fruits of tomato show all of the common features of the berries. Weier, *et al.* (1982) reported that tomato fruits develop from the ovary of the pollinated flower. Due to the pericarp walls, the tomato fruit is fleshy. The tomato fruits contain hundreds of seeds. Except for cherry tomatoes, most of cultivated tomato varieties have 4 or 5 locules. The locules are bounded by the pericarp. The pericarp contains the inner wall, the radial wall, and the outer wall. The placenta and the pericarp contain the fleshy tissue of the tomato. Locular cavities contain the seeds of the fruits and the seeds are covered in gelatinous membranes. Outer wall of the pericarp has vascular bundles and they travel from the stem to the center of the fruit (Ho and Hewitt 1986).

2.1.3. Common Tomato Diseases

Diseases of tomato are the main restricting production factor of tomato. Tomato diseases can be divided into two main groups. The first group comprises plant pathogenic microorganisms such as bacteria, fungi and nematodes viruses. These microorganisms are spreadable since they can spread from plant to plant in the field. Frequently their spread can be very fast when environmental conditions are suitable for these microorganisms. The second group comprises physical or chemical factors, physiological and nutritional disorders and chemical injury (Damicone, and Brandenberger, 1972). Non-infectious diseases cannot spread from plant to another; though, the disease distribution could be fairly even and widespread if the whole plant was exposed to the adverse environmental factor. The serious infectious diseases of tomato that cause commercial losses are bacterial spot, bacterial speck, bacterial canker, *Botrytis* gray mold, white mold, late blight, leaf mold, powdery mildew, *Septoria* leaf spot, tomato spotted wilt and other viruses

2.2: *Botrytis* Gray Mold

Botrytis gray mold is a fungal disease that infects a wide range of herbaceous annual and perennial crop species. There are several *Botrytis* species that cause blights. The most common of them is *Botrytis cinerea*. Droby and Lichter (2004) reported that cool, rainy spring and cool rainy summer conditions around 15 °C favor *Botrytis* infections. *Botrytis cinerea* can be mostly harmful when cool rainy, drizzly weather conditions remain for several days.

The fungus *B. cinerea*, a plant necrotrophic pathogen, causes grey mold. The fungal hyphae can penetrate through natural openings and wounds on the plant and they spread from previously infected dead tissues into healthy plant tissues. *Botrytis cinerea* is a main cause of postharvest rot of tomatoes at harvest and in the storage. Subsequently, *B. cinerea* is also able to infect at lower temperatures and cause important economic losses in either pre- or postharvest vegetable crops (Mansfield, 1980).

In addition to vegetable crops, *Botrytis cinerea* can infect numerous ornamental plants including: chrysanthemum, anemone, hydrangea, begonia, calendula, dahlia, dogwood, marigold, zinnia, fuchsia, hawthorn, heather, geranium, pansy, periwinkle, petunia, rose, violet, snapdragon and sunflower. The other damaging *Botrytis* blight fungi *B. paeoniae* and *B. tulipae* have strict host preferences infect tulip and peony, respectively. Williamson *et al.* (2007) reported that cucurbit, bean, beet, asparagus, carrot, crucifers, celery, chicory, eggplant, grape, endive, lettuce, pepper, onion, potato, rhubarb, rutabaga, raspberry, shallot, strawberry, tomato, turnip were well-known hosts of *B. cinerea*.

2.2.1. Taxonomy and Classification of *Botrytis*

Botrytis was recognized by Micheli as genera in 1729 and he listed it in his book "Nova Plantarum Genera." Initially, *Botrytis* was occasionally confused with *Sclerotinia* spp. but the difference between *Botrytis* and *Sclerotinia* were explained by Smith (1900) and Whetzel (1945) resolved the confusion in 1945. The genera were reworked by Hennebert (1973) and he identified 22 species, most of them had a restricted host range, such as *B. tulipae* that attacks on tulips, *B. fabae* that attacks on beans or *B. squamosa* that attacks on onion (Hennebert, 1973). *B. cinerea* is ubiquitous species that can infect

many crop species that can cause serious injury before and after harvest. The name of *B. cinerea* was given in 1801 by Persoon. In the first time, Drayton and Groves (1939) observed the occurrence of apothecia by *B. cinerea* confirming the systematic relationship between the two forms of the *Botrytis*.

The pathogen *Botrytis cinerea* causes serious losses in more than 200 crop species worldwide including Turkey. Generally, it damages occur on mature or senescent tissues of dicotyledonous hosts, however, it typically gains entry to young tissues at the earlier crop development stage and remains inactive for a long period before quickly rotting tissues when the environment is favorable and physiology of the host changes. Consequently, considerable damage is caused succeeding harvest of healthy crops and with the following transport to far markets where the losses of fruits and vegetables become obvious. However, *B. cinerea* also causes huge losses in some cultivated areas- and greenhouse-grown vegetable crops before harvest, or even at the sowing stage in some hosts. Some monocotyledonous host plant species are susceptible to *B. cinerea* attack, and there is a group of associated *Botrytis* species specialized to contaminate about a dozen of monocot host plants (Staats *et al.* 2005, 2007).

2.2.2. Life Cycle of *Botrytis cinerea*

Botrytis cinerea can produce mycelium, asexual spores (conidia), sexual spores and sclerotia during its life cycle. The mycelium comprises articulated filaments, grayish cylindrical, occasionally vesicular at the central partition. Its diameter differs markedly depending on the conditions for the hyphae development (Faretra and Antonacci, 1987; Faretra *et al.*, 1988). The mycelium on fruit produces clusters of gray conidiophores. Sometimes kind of growth may vanish and give a white mycelial that corresponds to the elongation of hyphae slender, hyaline that spread in the form of web (Beever and Weeds, 2004). The mycelium could be deposited in plant debris from the previous crop. Under favorable conditions *B. cinerea* grows well and gives conidia whose growth is characterized by the production of conidiophores. Holz, *et al.* (2004) stated that conidia relief is increased by a moist climate, and then they are distributed by wind, rain and insects. Under unsuitable conditions, mycelium and conidia sclerotia developments are occur. The conidia consist of combined white mycelium (Coley-Smith *et al.*, 1980). As conditions get older, they become black and hard. The conidia are consisted of a dense

cortex cells that can act barrier pseudo-parenchymal cells and a large central medulla composed of filamentous hyphae. The sclerotia germinate in the spring, and produce mycelium or conidia (Faretra, *et al.*, 1988; Beever and Weeds, 2004; Delcan and Melgarejo, 2002). *B. cinerea* overwinters as sclerotia or as mycelium in plant debris. *B. cinerea* can be seed borne as spores or mycelium in a few crop species. Other crops can be infection sources of *B. cinerea* and are possible to cross-infect. Williamson, *et al.* (2007) reported that conidia were airborne and could be spread on the surface of the plants by splashing rain drops. In the production areas, spores on the tomato plants can germinate and infect the plants when the enough humidity comes from rain, fog, dew, or sprinkler irrigation (Figure 2. 1). Dying and death flowers are a suitable site for fungal infection. However, fungal infections may also result from direct contact of plants to the infested wet soil or plant residues. Stem lesions can develop both in wounds and in infected leaves in the greenhouse. Elad, *et al.* (1997) reported that availability of external nutrients, such as pollens in the infection droplet could noticeably promote *B. Cinerea* infection.

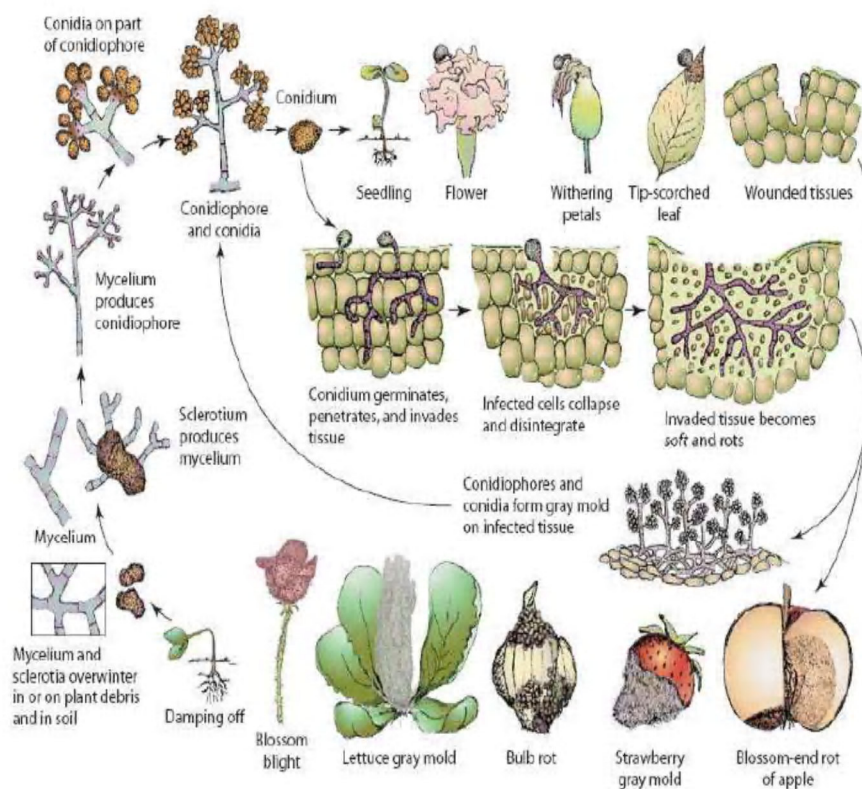


Figure 2. 1. Life cycle of *B. cinerea* and development of gray mold disease (Adapted from Agrios, 2005).

2.2.3. Pathogenicity of *B. cinerea*

Infection sources of *B. cinerea* are the conidia which are dispersed in the air. The conidia germinate after placing on the plant surface and short germ tubes are formed by the conidia that directly penetrate into the plant tissues. Previously established mycelia are good infection source, in this certain case, multi-cellular infection structures are formed. Generally, the epidermal and underlying cells die after penetration and *B. cinerea* establishes a primary infection. After that, *B. cinerea* starts an immense outgrowth which results finally in the soaking of the plant tissue and formation of conidia for new infections. *B. cinerea* produces phytotoxic metabolites such as reactive oxygen species, cell wall degrading enzymes, botrydial and botcinic acid, necrosis inducing proteins, peptidases and phyto-hormones during the interaction (Choquer *et al.*, 2007). Gene replacement methods were identified only a few virulence factors to date, probably due to their redundancy (Tudzynski and Kokkelink, 2009). Hereafter, the importance of the toxic secondary metabolites such as botrydial and botcinic acid for infection becomes apparent only when strains are lacking both toxins. The asexual macroconidia shaped by branched conidiophores are undoubtedly the major source of main inoculum under the given environmental conditions, such as high moisture and reasonable temperatures. However, for over-wintering the dark pigmented sclerotia serve as survival structures. Those pigmented sclerotia may germinate vegetatively to produce new mycelia and conidia, or they can behave as a female parent in the sexual reproduction when microconidia have opposite mating types. Apothecia, containing the linear asci with the ascospores, develop on the fertilized sclerotia (Faretra and Antonacci, 1987). It is not easy to estimate how often sexual reproduction occurs in the nature; yet, it is supposed that it greatly contributes to genetic variation of *B. cinerea*. When *B. cinerea* produces sclerotia and microconidia, it can act as a female or a male partner in the reciprocal crosses; but, not all isolates of *B. cinerea* form sclerotia (Giraud, 1997).

B. cinerea was seen to respond to the various wavelengths of light. Red light promotes sclerotial development, UV light promotes conidiation and blue light leads to undifferentiated aerial mycelia accumulation. It was hypothesized in 1975 that two receptors sensing UV/ blue light and red / far-red light were involved in regulation of asexual reproduction in *B. cinerea* (Tan, 1975; Schumacher, 2014).

Adaptation of *B. cinerea* to different environmental conditions has been well explored, and various adaptation strategies in the attacked host tissue were projected. These adaptation strategies of *B. cinerea* comprise hydrolytic enzymes that secreted during germination (Elad, 1997), other cell wall-degrading enzymes that have increased activity in the infected ripening fruit (Barkai-Golan, *et al.*, 1988), and active oxygen species that persuade cell death caused (Von Tiedemann, 1997).

Gray mold growth on the plant surface is related with an increase in ethylene production from the infected plant tissues that are most often ascribed to the plant host. Formerly, it was indicated that *B. cinerea* grown on different nutrient sources did not produce ethylene (Elad, 1990). This result opposes the later finding which ethylene was released by *B. cinerea* when it was grown on a defined medium (Qadir, *et al.*, 1997). The ethylene synthesis mechanism of *B. cinerea* is not well known. Lately, it was stated that ethylene was a principal marker for fruit pathogenesis. Cristescu *et al.* (2002) reported that some other infection-related plant products were markers of *B. cinerea* pathogenesis.

2.2.4. Symptoms of *B. cinerea*

Stem lesions on tomatoes seedlings can occur at the soil surface. Stem entry can occur through senescent cotyledons or damaged plant tissue. Also, stem lesions can occur during the later growth stage of the crop plants. Stems can become infected through dead leaves, leaf scars, or any form of stem damage. Stem lesions often somewhat girdle the stem, but occasionally the whole stem is affected, and the plant is killed. Petiole lesions seem very similar to those on the stem and often result from infection and colonization of a leaflet.

Tissue senescence or any physical or chemical damages of the leaflets are the first seen lesions. The pathogen can grow along on the leaf petiole to the main stem and can finally form lesions on stems and petioles. Fallen flower parts on the leaves are good beginning point for leaflet fungal colonization. Pollens and other flower parts can also perform as a good starting point. *B. cinerea* infection, not only they stimulate germination, but also increase the virulence of *B. cinerea*. In the production areas, *B. cinerea* look as if a gray, velvety covering of spores on the surface of dying flowers (Fig. 2.2 A) and on the calyx of the fruits (Figure 2.2 B). Infected flowers and

fruit are good source of infections and infections spreads back toward the stem and the stem turns light brown to white and a canker develops (Figure 2.2A). Immature green fruits turn beige or white, when they touch the infected parts of the plant. A soft rot can occur on the fruit skin and then the inner tissue turn into mushy and watery structure (Figure 2.2 C). Later, a gray fuzzy mold develops (Figure, 2.2 D), and sclerotia can be seen (Figure 2.2C). If this stage is seen in the greenhouse, the ground of the greenhouse will be covered with fruits that are aborted from the plants. In the production areas, the passageways can be covered with discarded fruits (Figure, 2.2 E). Also, green fruits may be infected directly by airborne spores rather than by contact contamination with other infections. White circular spots occur on the fruits and they have named "ghost spots." These spots will keep on green on the mature fruits (Figure 2.2 F). As fruit matures the color of the coronas changes from white to yellow. The "ghost-spot" symptom is due to the spore germination and their penetration on the fruits that is only vulnerable to attack up to cherry size. Immediately the surface of the fruit is getting shiny, it is no longer susceptible *B. cinerea* infection. Penetration of *B. cinerea* mycelium into the fruit develops a host reaction stopping any further mycelial growth and results in localization of the pathogen.



Figure 2.2 Stages of gray mold disease development.

Great amount asexual spores of *B. cinerea* can be produced when the conidia establish a plant surface. They germinate and form an appressorium and peg penetration breaches the plant surface cuticle. Because the appressorium cannot be detached from the germ

tube by a septum; it is unlikely that adequate turgor can be made to affect entry by physical force alone. Enzymes such as cutinases and lipases are produced. The tip of the penetration peg produces hydrogen peroxide. When the cuticle has been penetrated, the penetration peg touches an epidermal cell and often grows into the pectin-rich cell wall that is perpendicular to the plant surface. Zitter (1986) reported that plants having low pectins in their cell walls were poor hosts for *B. cinerea* that has effective pectinolytic machinery.

Botrytis cinerea produces various low-molecular weight arsenals metabolites which cause host death. During penetration of the cuticle and formation of the primary lesions, *B. cinerea* activates an oxidative burst from the plant, accumulates free radicals and makes hypersensitive cell death in the host tissue. Although this shows resistance against biotrophic pathogens such as *B. cinerea*, programmed plant cell death is beneficial to necrotrophs, because biotrophic pathogens feed on the dead cells of the hosts. The fungus is also able to inhibit host immunity by production of small RNA (sRNA) molecules that can cause gene silencing. As well as the above-mentioned pectinases, *B. cinerea* produces enzymes such as cellulases and hemicellulases to decay plant cell walls to obtain nutrition (Boddy, 2016).

2.2.5. Epidemiology of *B. cinerea*

B. cinerea overwinters as sclerotia or as mycelium on death plants and it may be seed born as spores or mycelium in a few crops. Several crops can be infection sources of the pathogen. Conidia are airborne, and they can be carried with the splashing rain drops. High relative humidity is required for production of the prolific spore. In the production areas, spores landing on surface the tomato plants germinate and infect the plant when enough humidity on the plant surface comes from rain, dew, fog, or irrigation. Optimum infection temperatures for *B. cinerea* are between 18 and 24 °C, and infection can occur within five hours. Higher temperatures, above 28 °C inhibit growth and spore production of *B. cinerea*. Dying flowers are a good infection site for *B. cinerea*; however, infections can be occurred due to direct contact with infested wet soils or plant debris. Stem lesions may develop either by direct colonization of *B. cinerea* in the wounds or over the infected leaves. Infections can remarkably increase due to the presence of external nutrients in the pollen grains. The wound type is thought to effect

development of the stem lesion. The leaf breaking off is stated to give a lower incidence of stem lesions than cutting off leaves with a knife, leaving a stub. (Zitter, 1986)

2.2.6. Management

Disease resistance or susceptibility of a given crop plant is determined by the plant-pathogen combination and the developmental stage of the host plant tissues. The maturing process of fleshy fruits is an example of a progressive conversion which overlaps with increased susceptibility to pathogens. Giovannoni (2004) stated that ripening of the fruits requires a complex network of hormone-controlled pathways leading to important changes in the biochemical and physiological properties of the fruits. During the ripening events, cell wall structure modifications, starch conversions into simple sugars, apoplastic pH changes and antimicrobial metabolites decrease contribute to susceptibility of the fruit to the pathogens (Prusky and Lichter, 2007; Cantu *et al.*, 2008). The increased susceptibility of mature fruits to pathogens can be a default outcome of ripening and increased susceptibility can be aided by the ripening processes (Cantu *et al.* 2009).

Pathogens of the fruit shows biotrophic, necrotrophic, and hemibiotrophic life styles (Prusky and Lichter, 2007; Cantu *et al.*, 2008) that shows diverse infection strategies (Glazebrook, 2005). Necrotrophs, such as the ascomycete cause necrosis via synthesizing hydrolytic enzymes (Van Kan, 2006), secreting toxins (Govrin *et al.*, 2006; Dalmais *et al.*, 2011) or capturing the enzymatic machinery of the plants (Cantu *et al.*, 2009). Therefore, the infection strategies of many pathogens can challenge to the host plant competency and complexity and decrease effective defense mechanisms.

2.2.6.1. Resistant cultivars

There are no known tomato varieties reported to resistance to the gray mold.

2.2.6.2: Cultural control

Cultural control methods that help aeration and drying of plant canopy after rain, while keeping sufficient water supply to the roots, are the most effective methods to prevent *Botrytis* infection (Elad and Shtienberg, 1995). Due to increased environmental concern and human health problems, some alternative potential of chemical fungicides was

addressed as ecological control measures such as microbial antagonists that are capable of disease suppression (Dubos, 1992). Cultural control measures can be potent powerful methods to reduce plant diseases in greenhouses where the high value cash crops is grown and the farmers make great efforts during the cropping season. Such methods are commonly aimed at changing the microclimate in the canopy and around the susceptible plant organs, prevention of inoculum entrance into the greenhouse and its build up and rendering the host plants less susceptible to diseases. *Botrytis* susceptibility can be reduced by calcium loading of the plant tissues and alteration of nitrogen fertilization (Elad and Shtienberg, 1995). Resistance to *B. cinerea* has been not reported yet. In strawberry, some control measures such as combination of one-row-system and leaf and fruit sanitations reduce *Botrytis* mold damage in the first crop year compared with a two-row-system without fruit and leaf sanitation (Schmid *et al.*, 2005).

2.2.6.3. Chemical control

One of the most common methods of *Botrytis* control is the application of synthetic chemical fungicides. Spraying of chemical fungicides directly to sporulating wounds can be practiced. Nevertheless, fungal activity of several fungicides can be lost, because of resistant development. While fungicides continue an essential method to control *B. cinerea* infections, it is important to observe the populations of the *B. cinerea* for its resistance against known fungicides (Elad and Shtienberg, 1995). Chemical control remains the major way to decrease the occurrence of *B. cinerea* and other *Botrytis* diseases on the major crop plants. The use of fungicides is an effective way to protect crops against *Botrytis* spp. spraying aerial parts of plants with fungicides is the most common interventions. The applied fungicide doses vary from 2000-3000 g/ha (e.g. maneb, thiram, dichlofluanid) to 400-500 g/ha (e.g. carbendazim, fludioxonil, pyrimethanil). The number of treatments during a season varies from one or two, to more than twenty. Fungicides are available to control *B. cinerea* on tomato; however, they may only be used once cultural. Fungicide-resistant *B. cinerea* has been reported on many crop plants. In order to prevent resistant development, apply fungicide according to labeled instructions and rotate different fungicide groups or mix the different group of fungicides to prevent development of fungicide-resistant *B. cinerea*.

2.2.6.4. Biological control

Biocontrol of gray mold diseases caused by *B. cinerea* has been extensively studied over the last 60 years, since resistance to several known fungicides has developed (Moorman and Lease, 1992). There is an increased concern about the need for finding effective fungicide against the pathogen. Some chemical formulas cannot be registered due to the development of pathogen resistance in a limited space. Furthermore, many fungicides labeled for *B. cinerea* on tomato are under inspection as potential carcinogens. Future availability of fungicides use on vegetables may be limited due to the environment concerns and human health. The occurrence of fungicide resistance and growing consumer demands for reduced chemical residues on the fruits highlights the requirement of various disease control approaches.

Use of biological agents is an alternative way to control of *Botrytis* gray mold disease. Biocontrol of *Botrytis* gray mold disease with bacteria, yeasts and filamentous fungi was well studied over the last two decades (Tronsmo, 1991, Blakeman, 1993; Elad and Shtienberg, 1995). Biological control offers a charming alternative or supplement to the use of synthetic chemicals for gray mold control. Because microbial biocontrol agents are supposed to be less demanding to the environment and their complex mode of action decreases the risk of resistance development. For instance, *Ulocladium atrum* exhibited an actual potential to protect stem wounds from *B. cinerea*. The level of protection supplied by the microbial biocontrol agents was as good as that supplied by the chemical fungicide in heated and unheated greenhouses and plastic tunnels (Fruit and Nicot, 1999). Studies with the antagonistic strains of *Fusarium* sp in 16 greenhouses in the southern France showed significantly reduced incidence of stem lesions caused by *B. cinerea* on tomatoes (Decognet *et al.*, 1999).

It was determined that *M. dimerum*, *L. lecanii* and *R. sachalinensis* extract are compatible for application on tomatoes when they were combined together to control *B. cinerea* (Bardin *et al.*, 2004). Yeast isolates reliably decreased disease incidence and sporulation of *B. cinerea* on tomato plants. Several isolates decreased disease by more than 75% in all experiments (Dik *et al.*, 1999). *U. atrum* was found the most effective for suppressing *Botrytis* spp. Colonization strawberry fruit rot was significantly decreased by weekly applications of *U. atrum* in 3 out of 4 field experiments. The

antagonist was as effective as fungicide treatments (Kohl and Fokkema, 1998). *U. atrum* is a strong competitor on necrotic above-ground plant tissues. In onion leaf spot the potential of the antagonist to reduce colonisation of necrotic leaf tissue by *Botrytis spp.* and subsequent sporulation was studied in two field experiments. *U. atrum* colonised necrotic tissues and consistently reduced the sporulation of fungal competitors (Kohl *et al.*, 2003).

The effects of *Trichoderma harzianum* on the suppression of *B. cinerea* was determined Ten days after inoculation with *B. cinerea* and *T. harzianum*, the incidence of infected stem pieces the intensity of sporulation was greatly reduced (O'Neill *et al.*, 1996). Biocontrol efficacy of the yeasts to control gray mold and blue mould was successful for apples and pears (Tian *et al.*, 2002).

2.3. Plant essential oils

Resistance problem of known drug is one of the most important problems to consider development of new antimicrobials. Plant essential oils are candidate as a promising good alternative to known antibiotics, and up to now, numerous plant essential oils were reported to have significant antimicrobial activities. The mode of action of essential oils is attributed to the phenols or oxygen-substituted derivatives. The antifungal, antibacterial, antiviral, antinematicidal, antiseptic, and other therapeutic applications of essential oils are well known since the prehistoric times (Gurib-Fakim, 2006). According to the World Health Organization, about 80% of the World population, especially in the developing countries, depends on medicinal plants and their extracts for main health care (Valiakos *et al.*, 2015). The modern pharmaceuticals, cosmetics, and food industries are established on the information of the medicinal plants that can be used to treat infectious, systematic, and inflammatory diseases to preserve food and to produce perfume (Hammer *et al.*, 1999).

2.3.1. Historical

In the ancient world, medicinal and aromatic plants were widely used by rhizotomists. The Egyptians, Chinese, Indians, Arabs and Romans were used medicinal plants, to treat several diseases and they built philosophical principles, traditions, and practices in their culture. Hippocrates (late 5th century BC) talk about 300-400 medicinal plants

(Lardos, 2006). Plant essential oils are mentioned on clay tablets of cuneiform writing (2600 BC) in Mesopotamia (Gurib-Fakim, 2006). The great civilizations and empires controlled the production and trade of medicinal plants for centuries. In the middle ages the cultivation of medicinal plants in monasteries and abbeys accelerated the development of knowledge on their therapeutic properties. However, traditional use of medicinal plants was gradually abandoned in the developed world when the modern medicines were used to begin. Though, medicinal plants and their extracts gained great attention to use them instead of the known synthetic chemicals in the modern drug and food and industries (Cowan, 1999).

2.3.2. Composition of Essential Oils

Paracelsus used the term essential oil in first time at the beginning of 16th century, who named the effective component of each drug “quinta essentia” (Edris, 2007). The essential oil components cover almost 500 compounds, including terpenes, terpenoids, and aliphatic and aromatic compounds such as phenols and aldehydes (Sakkas and Papadopoulou, 2017).

Essential oils are obtained from the essential oil bearing plants by hydro distillation, fermentation, crushing, extraction, and airing, however, steam distillation is the most common method.

Distillation is used an efficient essential oil extraction method by the Egyptians, Indians, and Persians in the ancient time and it was improved by the Arabs in the 9th century AD (Burt, 2004).

The whole components of essential oils can be determined by chromatographic methods, mostly with the use of gas chromatography–mass spectrometry (GC-MS), thin layer chromatography and high-performance liquid chromatography (Sakkas and Papadopoulou, 2017).

There are about 3000 known essential oils, of which 300 are widely traded in the world market (Freires, 2015). Different factors such as environmental conditions, the soil properties and cultural methods, the climate and harvesting date and time, the drying

and storage and processing conditions, the oil extraction methods, and analysis of the chemical components affect the composition of the essential oils.

2.3.3. Bioactivities

Over the past decades, the development of drug-resistant pathogens takes great attention on essential oils due to their high antifungal, antibacterial, antioxidant potentials and bio-regulatory actions (Burt and Reinders, 2003; Holley and Patel, 2005).

Because of the great biological and structural variation in essential oil components, they are supposed to constitute a renewable and unique source of required novel antimicrobial chemicals. Bearing in mind of the diverse groups of essential oil components, it is most possibly that antimicrobial activity of essential oils is not attributable to only one mechanism; however, there are numerous modes of actions of essential oils on the microbial cell. Hydrophobicity, disruption of the electron flow, disturbance of the cytoplasmic membrane, coagulation of cell contents and active transport can be assumed as possible mode of action of essential oils on microorganisms (Burt, 2004). Guynot *et al.* (2003) stated that other mode of actions such as the electric potential of the proton motive force can cause membrane malfunction and disturbances of the pH gradient. Walsh *et al.* (2003) reported that essential oils having the great antibacterial properties had higher phenolic compound percentages such as thymol, carvacrol and eugenol that cause structural and functional disturbances in the microbial cell membrane, and effect on cellular proteins that stuck into the cytoplasmic membrane. The Mediterranean regions have the naturally growing area of essential oil bearing plants such as Origanum (*Origanum syriacum* var. *bevanii* L.), lavender (*Lavandula stoechas* L. subsp. *stoechas*), zahter (*Thymra spicata*) and rosemary (*Rosmarinus officinalis* L.) that are rich in indigenous aromatic and medicinal plant compounds. Nowadays, they are cultivated for their essential oils for their medicinal and aromatic and antimicrobial properties (Soylu *et al.*, 2010).

2.3.4. Oregano Essential Oil

This herb oregano belongs to the Lamiaceae family and *Origanum vulgare* is the main representative of this species. Etymologically, the name *Origanum* comes from the Greek name (origanon), that originated from the words (oros = mountain) and (ganos =

brightness, beauty), because oregano grows at altitudes of 400-1800 m above the sea level and sunny places. The *Origanum vulgare* contain essential oil between 0.5 and 7%, and the major essential oil components are carvacrol and thymol, as well as their precursor monoterpenes p-cymene and γ -terpinene at a lower proportion (Kokkini *et al.* 1997).

The genus *Origanum* covers several morphological and chemically diverse species, and most of them are naturally grown in the Mediterranean region. One of the most known widely utilized oregano species are *O. vulgare* with the subspecies *O. vulgare* subsp. *L. vulgare*, *O. vulgare* subsp. *L. glandulosum*, *O. vulgare* subsp. *L. gracile*. Closely related species are *O. onites*, *O. syriacum*, *O. minutiflorum*, *O. majorana*, growing in Western Asia, and Eastern Mediterranean contains carvacrol, p-cymene and γ -terpinene at the various concentrations. *O. vulgare* subsp. *hirtum* (Greek oregano) and *O. onites* (Turkish oregano) are the most known commercial oregano species (Gounaris *et al.*, 2002).

Since antiquity, oregano has been a widespread usage, and its essential oil is well recognized for its antiseptic and antispasmodic effects. The studies on the antioxidant properties of the oregano essential oil showed that it had great antioxidant power with remarkable effects in inhibiting fat oxidation due to its great thymol and carvacrol content. McKay and Blumberg (2006) stated that oregano essential oil contain highly bioactive compounds that had insecticidal and acaricidal effects. Oregano essential oil at 12.8 $\mu\text{g/ml}$ concentration completely inhibits *B. cinerea* growth *in vitro* study and they stated that oregano essential oil was the most efficient essential oil under greenhouse conditions against grey mold (Soylu *et al.*, 2010).

2.4. Nanotechnology

Nanotechnology is a constantly evolving modern scientific field of theoretical and applied science and technology. The term 'nanotechnology' is based on the prefix 'nano' which comes from the Greek word meaning dwarf or extremely small. More exactly; the word 'nano' means 1×10^{-9} or one billionth part of a meter (Stylios, 2005). Nanoparticles, the building blocks of nanotechnology are described as particles having more than one dimension ranging between 1 and 100 nm. Nano-structured materials are anticipated to offer better built, longer-lasting, cleaner, safer and smarter products for

the home, communications, medicine, transportation, agriculture and other industries in general. Nanoparticles generally have unusual physical, chemical and biological characteristics that are absolutely different from their mother materials and individual molecules (Li *et al.*, 2001). Nanomaterials may have a significant role in stimulating sustainable agriculture and healthy foods globally (Gruère, 2012). Currently, nanotechnology has great influence on medicine biotechnology, energy, manufacturing industry, information technology. It is assumed that nanotechnology will bring new industrial revolutions.

Nanotechnology has great important potential to enhance agricultural productivity, biotechnology, precision agriculture, genetics, plant breeding, plant protection, fertilizer technology, and other related areas (Sastry *et al.*, 2010; Jha *et al.*, 2011). The majority of nanotechnology applications in agriculture have concentrated on the importance of the nanoparticles for increased efficiency and productivity. The nanomaterials are used in crop production and crop protection (Bouwmeester *et al.*, 2009; Emamifar *et al.*, 2010; Nair *et al.*, 2010; Sharon *et al.*, 2010)

2.4.1. History of nanotechnology

Plant pathologists are trying to invent effective nanomaterials to protect crops and agricultural products from bacteria, fungal and viral agents. It was stated that nanotechnological products such as nanobiological sensors, nanoscale platforms, miniature detection devices and nano sensors could play an important role in the pathogen inspection and disease control. Bordes *et al.* (2009) and Bouwmeester *et al.* (2009) stated that nanomaterials and nano-biocomposite materials have useful properties such as thermal stability, permeability, crystallinity, solubility, and biodegradability.

Nanomaterials and nano-biocomposites have versatile properties such as permeability, thermal stability, solubility, crystallinity, and biodegradability (Bouwmeester *et al.* 2009; Bordes *et al.* 2009). A number of nanotechnologies can offer an alternative control measures to existing crop control protocols in the short to medium term (Pérez-de-Luquend Rubiales, 2009). The nanoparticles have a great importance in the control of pathogens as compared to synthetic fungicides (Park *et al.*, 2006). Both nano-zinc oxide (ZnO) and nano-magnesium oxide (MgO) is an operational antibacterial agent

(Shah and Towkeer, 2010) due to their ease in dispensability, optical transparency and smoothness. Aruoja *et al.* (2009), Sharma *et al.*, (2009) and Huang *et al.* (2006) recorded that nano ZnO and MgO were an excellent anti-microbial protectant for food or food products.

2.4.2. Classification of Nanoparticles

Nanoparticles are basically divided into different categories depending on their size, morphology and surface charge. Based on some chemical and physical properties of the most known classes of nanoparticles are given below.

Carbon-based nanoparticles: Carbon based nanoparticles are completely made of carbon. Fullerenes, graphene, carbon nanotubes, carbon nanofibers and carbon black are carbon-based nanoparticles. Fullerenes comprise nanomaterials which are spherical in shape and made up of carbon atoms held together by sp^2 hybridization. Graphene, an allotrope of carbon, is a hexagonal network of honeycomb lattice containing carbon atoms in a two-dimensional planar surface. Graphene has around 1 nm thickness in the sheet. Carbon nanotubes are based on a graphene nanofoil with a honeycomb lattice of carbon atoms. Carbon nanotubes have nanotubes with varying diameters as low as 0.7 nm for a single layered and 100 nm for multi-layered and their length varies from a few micrometers to several millimeters. The ends of nanotubes can be either open or closed. Carbon nanofibers are produced from the graphene nanofoils with a cone or cup shape.

Metal nanoparticles: Nanoparticles that are purely synthesized from metal precursors to nanometric sizes either by destructive or constructive methods are metal based nanoparticles. All known metals can be synthesized into their nanoparticles. Aluminum, cadmium, cobalt, copper, gold, iron, lead, silver and zinc are the well-known metals for nanoparticle synthesis. Because of the well-known confined surface plasmon resonance characteristics, metal nanoparticles have unique optoelectrical characteristics. Alkali and noble metal nanoparticles such as Ag and Au have a wide absorption band in the visible zone of the electromagnetic solar spectrum. Metal nanoparticles find applications in many researches because of their advanced optical properties.

Ceramic nanoparticles: Ceramics nanoparticles are inorganic solids such as carbides, phosphates, oxides and carbonates of metals and metalloids such as calcium, titanium, silicon. They are synthesized via heat and successive cooling. They can be found in amorphous, polycrystalline, dense, porous or hollow forms. Ceramic nanoparticles have a wide application ranges since they have a number of favorable characteristics such as high heat resistance and chemical inertness.

Semiconductor nanoparticles: Semiconductor nanocrystals are produced from a variety of different materials. The size of the semiconductor materials is between 1 and 10 nm that they have metals and nonmetals characteristics. Therefore, they are used in a wide range of applications due to this property. Semiconductor nanoparticles have wide bandgaps and therefore showed significant alteration in their properties with band gap tuning. Therefore, they are very important materials in photo optics, photocatalysis and electronics due to their unique physical and chemical properties.

Polymeric nanoparticles: Polymeric nanoparticles are typically organic based nanoparticles that are generally nanocapsular or nanospheres in shape.

Lipid-based nanoparticles: These nanoparticles contain lipid moieties or other chemical components (approximately ranging from 10 to 1000 nm in dimension) that act to overcome biological barriers. Such as polymeric nanoparticles, lipid-based nanoparticles have a solid core made of lipid and a matrix comprises soluble lipophilic particles. They accumulate in or near diseased areas to functionally deliver the therapeutic agents for disease treatment or disease diagnosis. Lipid-based nanoparticles could be considered one of the best suitable vehicles to provide an integrated, personalized method for cancer diagnosis and cancer therapy in near future.

2-4-3 Synthesis of Nanoparticles

Different methods can be employed to the synthesis of nanoparticles, but these methods are divided into two main classes which are the bottom-up approach and top-down approach. These two approaches are further divided into different subclasses based on reaction condition, operation and adopted protocols as shown in Figure 2.4.

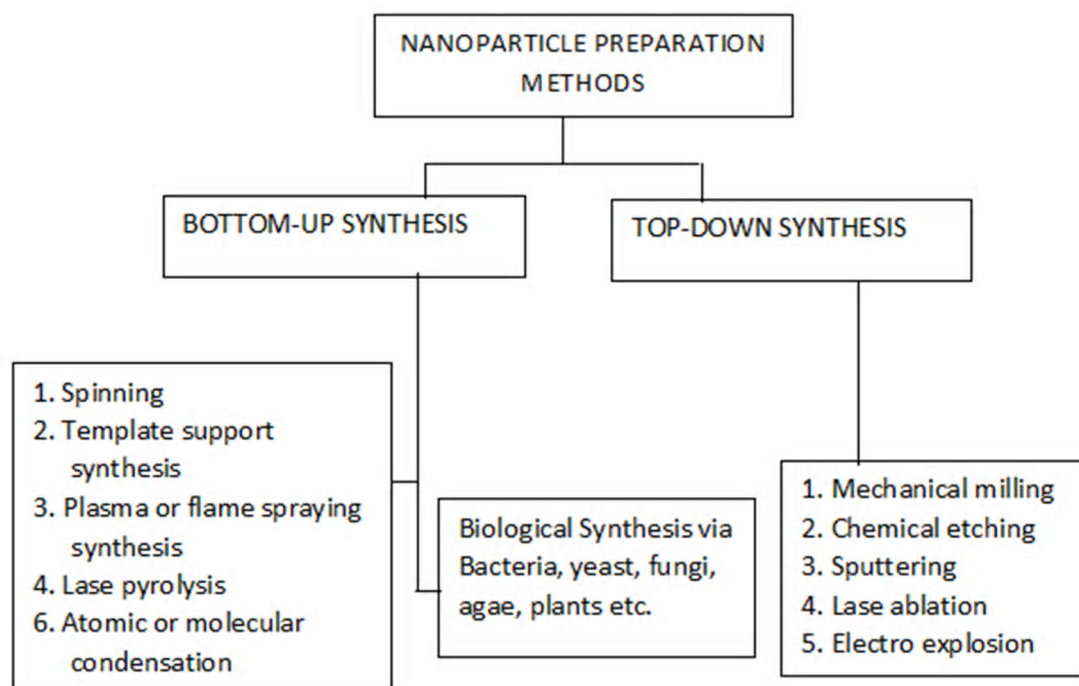


Figure 2.4. Methods for nanoparticles synthesis

2.4.4. Application of nanotechnology

Nanotechnology can contribute a sustainable growth in several areas of industrial application. The novel chemicals or physical properties of nano-scale particles provide useful roles that can be quickly exploited in biotechnology, electronics, medicine, material science energy sectors. These encouraging developments also push the agricultural sector to use nanotechnologies with the continuous innovation. Nanotechnology is strongly needed due to increase global food security and climate change contests. Formerly, agriculture was benefited from various technological innovations, such as synthetic chemicals, hybrid varieties, and biotechnological applications. At the present, researchers and farmers are now looking for nano-technological products as new source of agricultural improvements. Though, the food sector can be considered to be clearly benefiting from the nano-technological products (in food packaging, processing and distribution), its actual contribution to the agricultural industry is still ambiguous (Parisi *et al.*, 2015).

In Agriculture, nanotechnology can increase crop yield by breaking yield barriers through bio-nanotechnology, control pests and diseases, help to understand host parasite

interactions, accelerate the development of new generation pesticides, preserve food, strengthen natural fibers, remove soil and water contaminants, improve shelf life of fruits, vegetables and flowers and improve saline soils. Numerous researches on the application of agricultural nanotechnology has been continuing for largely a decade by now, seeking for solutions to several agricultural and environmental problems, such as improved cultivars, sustainability of crop production, and increased crop productivity. A number of authors have indicated the growing trend in patents and scientific publications in agricultural nanotechnology, especially for plant disease control and crop protection (Gogo *et al.*, 2012).

Table 2.1. The important applications of agricultural nanotechnology.

Application	Description	Example
Crop production and plant protection products	Nano-capsules, nanoparticles, nano-emulsions, viral capsids and as smart delivery systems of active compounds for plant pest and disease control	Neem oil (<i>Azadirachta indica</i>) nano-emulsion as larvicidal agent (Anjali <i>et al.</i> 2012).
Fertilizers	Nano-capsules, nanoparticles, and viral capsids for the enhancement of plant nutrition absorption by plants and the distribution of the nutrients to the defined cropping areas	Nano-fertilizers coated with zinc oxide (Milani, <i>et al.</i> , 2012).
Soil improvement and water retention	Nanomaterials such nano-clays and zeolites for water or liquid agrochemicals holding in the soil for their slow release to the plants	Soil enhancer products, such as nano-clay component, for water holding and release (http://www.geohumus.com/us/products.html).
Diagnostic nano-sensors and diagnostic devices	Nanomaterials such as sand fullerenes, nanofiber, electrochemically active carbon nanotubes which can be extremely sensitive biochemical sensors to carefully monitor plant health, growth and development and their environment	Pesticide detection with a liposome-based nano-biosensor (Vamvakaki, <i>et al.</i> 2007).
Nanomaterials from plant and microorganisms	Nanomaterials production through the use of genetically engineered plants or microorganisms and through the processing of agricultural waste products	Nano-fibers from wheat straw and soybean hulls for bio-nanocomposite production (Alemdar <i>et al.</i> 2008).

The aims of the nanotechnology in agriculture are to reduce the amount of sprayed chemical products by smart distribution of active ingredients, to decrease nutrient losses in fertilization and to increase yields through improved irrigation and nutrient management. Nanotechnology derived strategies can be explored in the field of plant breeding and genetic transformation (Torney *et al.*, 2007). Furthermore, agriculture can be a source of bio-nanocomposites with improved physical-mechanical possessions based on conventionally harvested materials, like wheat straw and soybean hulls, for bioindustrial purposes (Alemdar *et al.*, 2008). The most important agricultural nano technological applications are provided in Table 2.1.



CHAPTER 3

MATERIALS AND METHODS

3. 1. Plant Materials and Growth Conditions

Local tomato cultivar Karabacak was chosen for the present study due to its susceptibility to *B. cinerea*. The seeds were planted in 3 L plastic pots filled with soil, sand and vermiculite mixture in the ratio of 2:1:1. Four grams of ammonium sulfate, superphosphate, and potassium sulfate was applied in each pot. After emergence, the seedlings in each pot were thinned to one. The plants were irrigated with tap water when irrigation was needed.

3.2. Sample Preparation and Characterization of Nanomaterials

The nanomaterials, ZnO and CuO, were purchased as dry powders. Both nanomaterials will be suspended in deionized water at concentrations of 50 and 200 mg/ml for the stock suspensions, followed by sonication for 30 min to produce the required concentrations for subsequent experiments.

3.3. Fungal Strain and Growth Conditions

The *B. cinerea* standard strain used in this study was provided by Prof. Dr. Soner SOYLU (Mustafa Kemal University). The isolate was routinely cultured on potato dextrose agar that induced prolific conidia production for 7 days at 22°C. *B. cinerea* conidia were harvested from 7- to 14-day-old fungal plates in 20 ml of water. The suspended conidia were washed with sterile, deionized water. The suspended conidia were then diluted with sterile, deionized water to a final concentration of 10^5 conidia/ml in potato dextrose broth medium, with different nanomaterials.

3.4. Essential oil source and essential oil analysis

Aerial parts of oregano (*O. vulgare subsp. vulgare*) plants were collected at the flowering stage from Erciyes University Experimental Farm. Essential oil was extracted by water distillation for 3 h from air-dried leaves, by using a Clevenger-type apparatus.

The stems, leaves, and flowers of the oreganoplant were dried in the shade. The essential oils were extracted by water distillation for 3 hours from the dried leaves using a Clevenger type apparatus. The extracted essential oils were dried over anhydrous sodium sulfate and kept at 4°C in a refrigerator until analyzed. A Hewlett-Packard 2890 GC with FID was used to analyze. A HP-5 MS capillary column (30 m x 0.25 mm i.d. 0.25 µm film thickness) was used. Helium was the carrier gas (1.4 ml/ min). The column temperature programming was as follows: five minutes at 45°C, then at 3°C per minute up until 220°C and held for 10 minutes. The injector temperature was 220 °C and the detector temperature was 250°C. Injection was performed using the automatic settings. Essential oil samples (0.5 µl of the oil solution in hexane (1:100) were injected using the splitless technique into Helium carrier gas. Peak areas and retention times was determined by electronic integration. Essential oil GC-MS analysis was performed on Hewlett Packard 5970A mass selective detector (MSD), directly coupled to a HP 6890 GC. The column, temperature program and injection were performed using the same procedure outlined above. Injection was done using the automatic settings. The library search was done using “Wiley Library, WILEY275, NBS75K, NIST98, FLAVOR”. EI mass spectra were measured at 70 eV ionizations voltage over the mass range 10 - 400 u. Identification of the compounds was made through a comparison of retention times and mass spectra with the library standards (Stenhagen, 1974; Adams, 1995).

3.5. Antifungal Effect of Different Concentrations of Nanoparticles on Mycelial Growth *In vitro* Conditions

The nanomaterials, ZnO and CuO, were suspended in 20 percent potato dextrose agar medium to the final concentrations of 5, 50, 100, and 200 mg/l, poured into Petri dishes of 90 mm in diameter. Three potato dextrose agar plates of each nanomaterial concentration were tested. After cooling and solidification, a well-cultivated *B. cinerea* of 5 millimeters in diameter was injected into the medium's center, and the colonies

were moved to a room with a controlled 22°C temperature. The mycelia diameter was measured after an incubation period of 72 hours (Figure 3.1). The inhibitory effect (%) of nanoparticle and essential oil on gray mold was calculated using the following formula;

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100$$

where C is the diameter of the control treatment (mm), T is the diameter of either nanoparticle treated or essential oil treated colony (mm).



Figure 3.2. Measurement of fungal growth

3.6. Antifungal Effect of Different Concentrations of Nanoparticles and Essential Oils on Leaf Disc *In vitro* Conditions

The nanomaterials of ZnO and CuO were suspended in four percent water-agar to their ultimate concentrations of 50 and 200 mg/l. Each nanoparticle was mixed alone and with different concentrations of essential oil (0, 10, 20, 40, 80, 160, 320 and 640) µl/l. Tween-20 was incorporated into the agar medium to enhance essential oil solubility and allowed to solidify. For inoculation, tomato leaves were harvested from the greenhouse and brought to the lab (Figure 3.2). Discs of 1 cm diameter were excised from the leaf center with a hole-punch. They were rinsed using deionized water and placed on four percent water-agar plates that either contained the nanomaterials or did not. The leaf discs were placed in potato dextrose agar media that contained different nanoparticle concentrations before the suspension of conidia (2 µl, 10⁵ conidia/ml in potato dextrose

broth medium) independently containing different nanomaterials were dropped onto the petals to find the nanoparticle effect on fungi infection. This was done to emulate the leaf's microenvironment through soaking the nanoparticle suspensions on both sides. The suspension of conidia and nanoparticles were also dropped onto the leaf disc to ensure even distribution. Commercial fungicide and procymidone were used in the leaf disc for the negative control and positive control, respectively, in the second and the third group. After inoculation, Petri dishes were immediately transferred to a room with a controlled temperature of 22°C. After 72 hours (three days), the infection results were recorded using photographs and the diameter of the resultant inhibition zones were measured, averaged and the mean values were calculated. The inhibitory effect (%) of nanoparticle and essential oil on gray mold was calculated using the following formula;

$$\text{Inhibition (\%)} = [(C-T)/C] \times 100$$

Where C is the diameter of the control treatment (mm), T is the diameter of either nanoparticle treated or essential oil treated colony (mm).



Figure 3.3. Leaf discs taken from the greenhouse grown tomato plants

3.7. *In vivo* Application of ZnO and CuO Alone and Mixed with Essential Oil on the Whole Leaf

Tomato leaves were selected on the greenhouse growing plants, and the leaves were washed with deionized water. When the water on the leaves evaporated 50 and 200 mg/ml concentration of nanoparticles, ZnO and CuO and their mix with 80, 160 μ L essential oil, were sprayed on each selected leaf. The fungal suspension of conidia (2 μ L, 10^5 conidia/ml in potato dextrose broth medium) was sprayed in the upper and lower surfaces of the leaves. The treated plants were then placed in a growth chamber, in which the temperature was set at 22°C and the moisture was adjusted to 90%. After 4th and 6th days of incubation, the leaf was photographed and gray mold disease of *B. cinerea* assessment was carried out. Gray mold incidence and severity (percentage of infected leaf) were determined on 10 plants. Disease severity was recorded during flowering stage based on a 0 to 100 % score where 0 % = no symptoms on the leaves and 100 % = all leaves in the plant infected. The experiments were conducted in the completely randomized design with three replications.

3.8. Statistical Analyses

The experiments were conducted in triplicate. Statistical analysis, a one-way analysis of variance followed by LSD test ($P \leq 0.05$), will be conducted using SAS for Windows, version 9.3; SAS Institute.

CHAPTER 4

RESULTS

4.1. Essential oil components of *O. vulgare*.

Table 4. 1 shows the essential oil components, retention time, retention index and percent area of *O. vulgare* essential oil.

Table 4.1. Essential oil components of *O. vulgare*.

Compound Name	Retention Time	Retention Index	Area %
Tanshindiol	3.01	814	0.03
α -Pinene	3.62	987	0.56
α -Thujene	3.69	983	1.51
1,5,5-Trimethyl-6-methylene-cyclohexene	4.33	870	0.10
Dotriacontane	4.61	782	0.03
Sabinene	5.12	933	0.24
10-Heptadecen-8-ynoic acid, methyl ester	6.06	825	0.07
Cyclopentasiloxane, decamethyl-)	6.25	951	0.61
α -Myrcene	6.47	986	1.77
α -Terpinene	6.86	983	2.25
Cyclohexene, 1-methyl-4-(1-methylethenyl)	7.39	921	0.23
Sabinene	7.65	945	0.18
Mebutamate	8.05	827	0.03
cis-Ocimene	8.53	992	0.88
β -Terpinene	8.80	992	14.69
Ocimene	9.05	888	0.19
Benzene, 1-methyl-2-(1-methylethyl)	9.63	977	3.60
Cyclohexasiloxane, dodecamethyl-	12.38	903	0.58
9-Octadecenoic acid	13.94	699	0.08
1-Octen-3-ol	16.11	907	0.19
Trans sabinene hydrate	16.53	961	0.34

Cycloheptasiloxane, tetradecamethyl	19.16	926	0.61
Cholestan-3-ol, 2-methylene	19.67	844	0.16
Linalool	19.85	896	0.14
9-Octadecen-12-ynoic acid, methyl ester	20.18	821	0.05
trans-Caryophyllene	21.33	952	0.64
3-Cyclohexen-1-ol, 4-methyl-1-(1-methylethyl)	21.72	925	0.28
Hexadecadienoic acid, methyl ester	22.56	803	0.07
10-Heptadecen-8-ynoic acid, methyl ester	24.01	855	0.08
Isobornylthiocyanoacetate	25.30	852	0.28
Hexadecamethylcyclooctasiloxane	25.61	886	0.73
Junipene	26.32	838	0.20
Cyclononasiloxane, Octadecamethyl	31.44	869	0.62
2-Myristynoyl pantetheine	32.94	798	0.05
1-Heptatriacotanol	34.79	855	0.11
Eicosamethylcyclodecasiloxane	36.65	804	0.47
9,10-Secochola-5,7,10(19)-triene-3,24-diol	40.96	876	0.04
Carvacrol	42.54	975	66.08
2,5-Octadecadiynoic acid, methyl ester	45.54	860	0.11
1H-Purin-6-amine, [(2-fluorophenyl) methyl]	46.13	819	0.29
15,15'-Bi-1,4,7,10,13-pentaoxacyclohexadecane	48.24	823	0.05
1-Monolinoleoylglycerol trimethylsilyl ether	50.45	742	0.24
Octaethylene glycol monododecyl ether	54.51	830	0.19

Forty-three essential oil components were detected from the analysis of oreganoessential oil (Table 4. 1). Six major essential oil components greater than 1% were detected from oregano essential oil. The major essential oil components were carvacrol (66.08%), β -terbinen (14.69%), benzene, 1-methyl-2-(1-methylethyl) (3.60%), α -terpinene (2.25%), α -myrcene (1.77%), α -thujene (1.51%).

4.2. Antifungal Effect of Nanoparticles on Mycelial Growth under *in vitro* Conditions

Variance analysis of nanoparticles on growth inhibition of gray mold was given in Table 4.2.

Table 4.2. Variance analysis of nanoparticles on growth inhibition of gray mold.

Variation Resource	S.D.	Sum of Square	Mean Square	F
Dose	3	9357.753	3119.251	35.42**
Treatment	1	958.452	958.452	10.88**
Dose x Treatment	3	9182.642	3060.880	34.76**
Error	88	7749.280	88.060	
Total	95	27248.13		

Variance analysis table showed that dose, treatment and dose x treatment interaction were significant in terms of growth inhibition (Table 4. 2).

Table 4.3. Growth inhibition of nanomaterials on gray mold

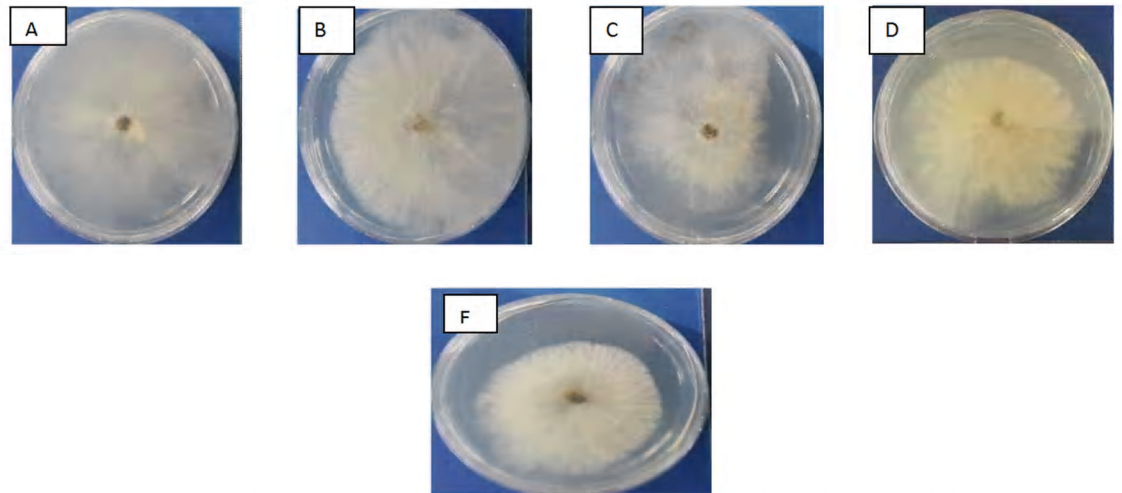
Nanomaterial	Growth inhibition (% of the control)
CuO	19.07 A
ZnO	25.39 B
LSD (% 0.05)	3.80

Compared with the control, growth inhibition difference between CuO and ZnO was significant (Table 4. 3). The growth inhibitions were 19.07 and 25.39 % for CuO and ZnO, respectively. Compared with CuO, ZnO had more inhibitory effect on gray mold growth.

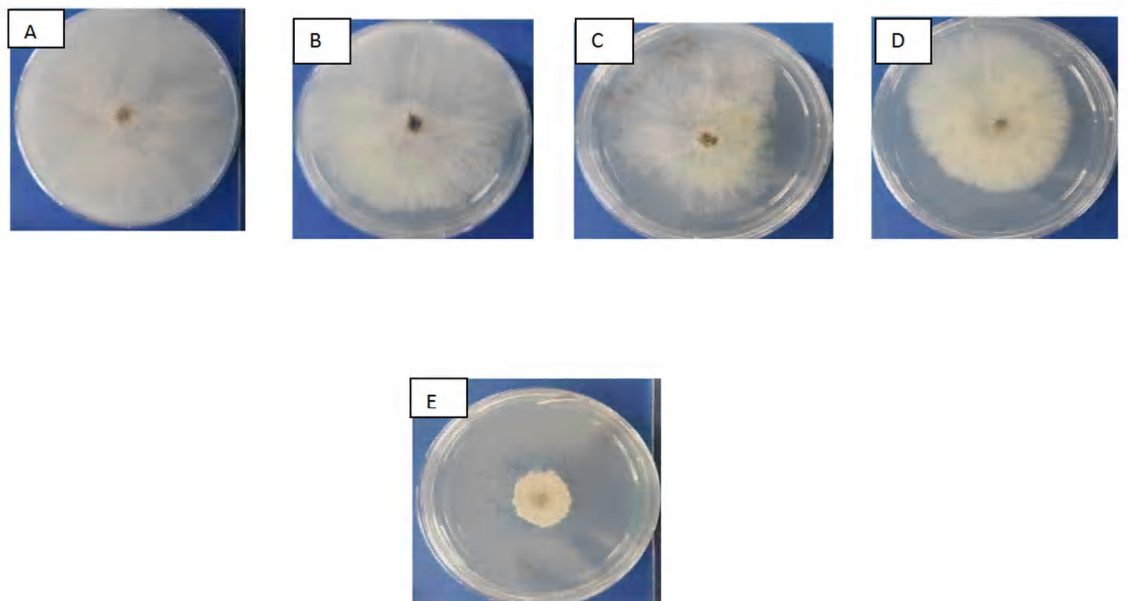
Table 4.4. Growth inhibition of nanomaterial doses on gray mold.

Dose (mg/L)	Growth inhibition (% of the control)
5	15.14 BC
50	14.58 C
100	20.32 B
200	38.89 A
LSD (% 0.05)	5.38

Growth inhibition of nanoparticles on gray mold varied among application doses (Table 4.4). Growth inhibition rates varied between 14.58 and 38.8%. The highest growth inhibition was obtained from 200 mg/l with 38.89 % and the lowest one was obtained 50 mg/l with 14.58 %. There was no significant difference between 5 and 50 mg/l application doses.



A :the control zero conc., B :5mg/l, C:50 mg /l, D:100mg/l, E: 200 mg/l (CuO)



A: The control zero conc., B:5mg /l, C:50 mg /l, D:100mg/l, E: 200 mg/l, (ZnO)

Figure 4.1. Mycelial growth of *Botrytis cinerea* on PDA.

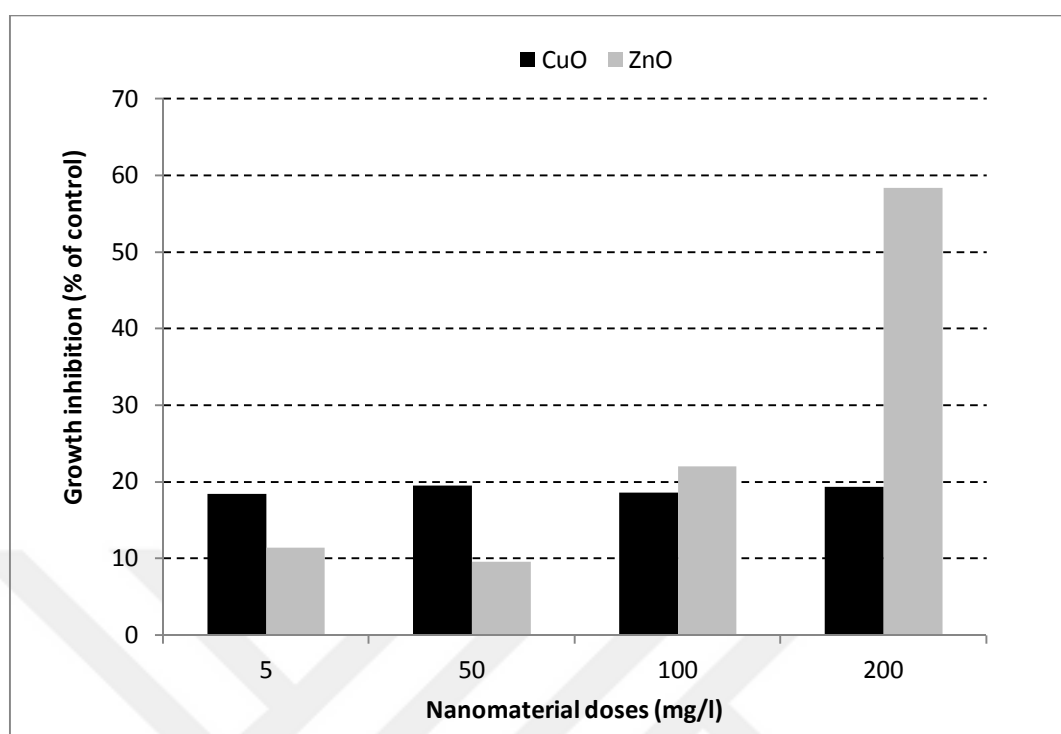


Figure 4.2. Effects of nanomaterial x dose interaction on growth inhibition of gray mold

In terms of growth inhibition, dose x nanomaterial interaction was significant (Figure 4.2). The significant interaction was resulted from growth inhibition of ZnO, since growth inhibition of ZnO was lower in the 5 and 50 mg/l doses while growth inhibition of ZnO was higher in 100 and 200 mg/l application doses. The growth inhibitions of CuO in 5, 50, 100 and 200 mg/l concentrations were not significant. Growth inhibition of CuO was not increased with the increased CuO concentration. However, growth inhibition of ZnO on gray mold increased with the increased ZnO concentration, except for 50 mg/l. It can be seen from the Figure 4.2 that different concentrations of ZnO caused different growth inhibition, but inhibition increased with the increase in the concentration of nanomaterials. However, this growth inhibition pattern was not observed for CuO. The maximum growth inhibition was found at the highest concentration 200 mg/ml. It was followed by 100 mg/l and 50 mg/l, doses of nanomaterials.

The nano ZnO had greater inhibitory effect on gray mold growth under *in-vitro* conditions, compared with CuO.

4.3. Antifungal Effect of Different Nanoparticles and Essential Oil on Leaf Disc under in vitro Conditions

Variance analysis of nanomaterials on oregano essential oil mix on growth inhibition of gray mold was given in Table 4.5.

Table 4.5. Variance analysis of nanomaterial and oregano essential oil mix on growth inhibition of gray mold.

	D.F.	Sum of Square	Mean Square	F
Nanomaterial (A)	1	1907.10	1907.10	49.93**
Nanomaterial dose (B)	1	796.30	796.30	20.85**
Oregano dose (C)	7	228023.58	32574.79	852.77**
A x B	1	173.18	173.18	4.53**
A x C	7	6297.80	899.68	23.55**
B x C	7	4490.08	641.44	16.79**
A x B x C	7	7380.27	1054.32	27.60**
Error	256	9778.87	38.19	
Corrected Total	287	258847.22		
C.V.			8.73	

Based on the ANOVA analysis for gray mold, when it was subjected to the nanomaterial and essential oil treatments, significant differences in the growth inhibition of the fungus were found (Table 4.5). It was clearly seen from the Table 4.5 that nanomaterial, nanomaterial dose, oregano dose, nanomaterial x nanomaterial dose, nanomaterial x oregano dose, nanomaterial dose x oregano dose and nanomaterial x nanomaterial dose x oregano dose interactions were significant.

Table 4.6. Growth inhibition of oregano essential oil doses on gray mold

Oregano essential oil doses (μ l)	Growth inhibition (% of the control)
10	37.28 E
20	50.83 D
40	65.27 C
80	86.40 B
160	100.00 A
320	100.00 A
640	100.00 A
LSD 0.05	2.86

Growth inhibition of gray mold was significant among oregano essential oil doses (Table 4. 6). Compared with the control, growth inhibition of oregano essential oil doses varied between 37.28 and 100.00 %. The lowest inhibition was obtained from 10 μ l essential oil application followed by 20 μ l essential oil application. Growth inhibition rate increased with the increased rate of oregano essential oil. Complete inhibition was obtained at 160 μ l and above essential oil applications.

Table 4.7. Growth inhibition of nanomaterial type on gray mold

Nanomaterial	Growth inhibition (% of the control)
ZnO	73.33 B
CuO	68.19 A
LSD 0.05	1.43

Compared with the control, growth inhibition rate of CuO and ZnO was significantly different (Table 4. 7). The inhibition rate of ZnO was 73.33 %, while the inhibition rate of CuO was 68.19 %. Nano ZnO found more effective on the control of gray mold than nano CuO.

Table 4.8. Growth inhibition of nanomaterial dose on gray mold

Nanomaterial doses(mg/l)	Growth inhibition (% of the control)
50	69.02 B
200	72.42 A
LSD 0.05	1.43

Table 4.8 shows that growth inhibition rate increased with the increasing nanomaterial dose. The growth rate inhibition was significantly higher in 200 mg/l nanomaterial dose than 50 mg/l nanomaterial dose.

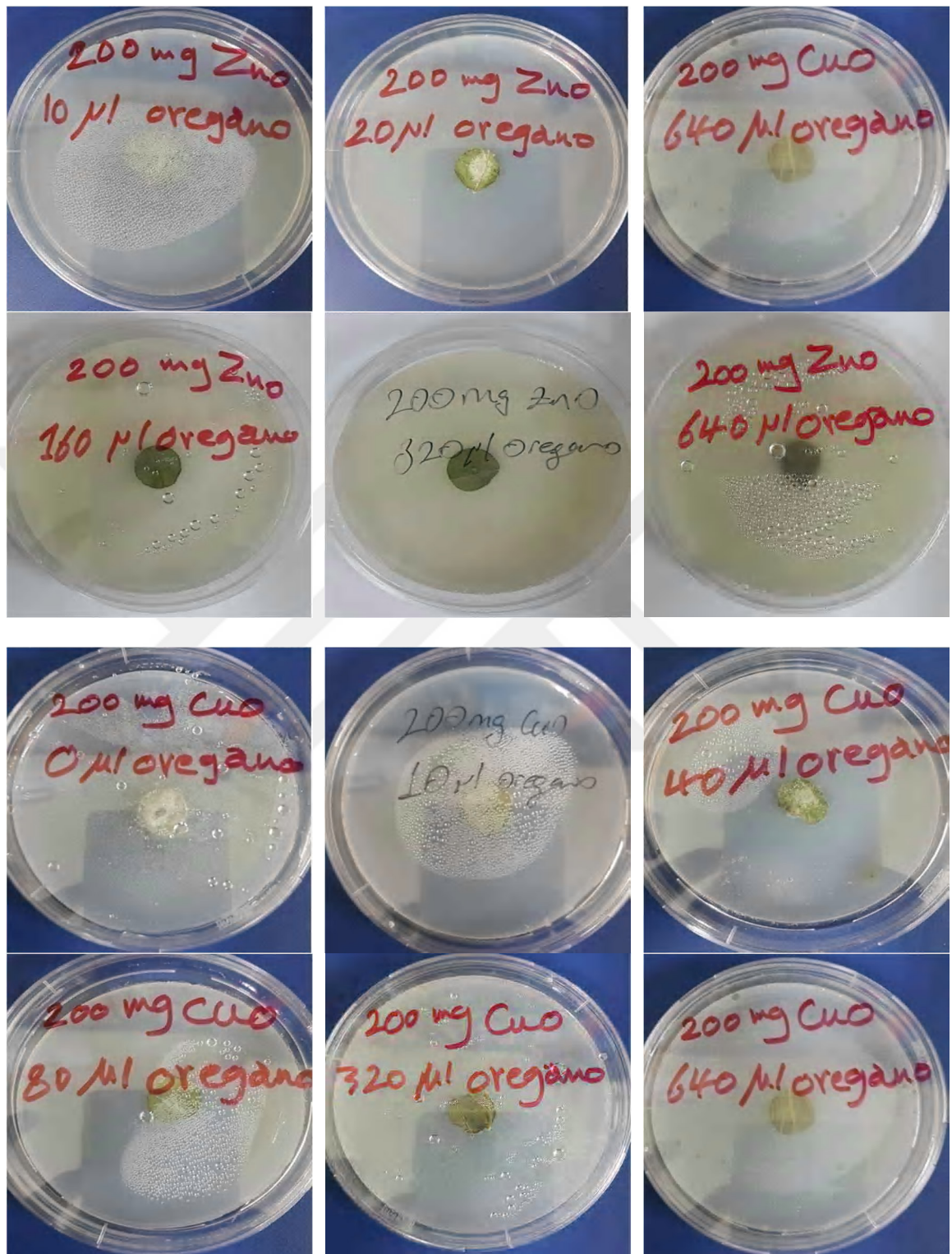


Figure 4.3. Fungal growth on inserted leaf disc of Potato DA.

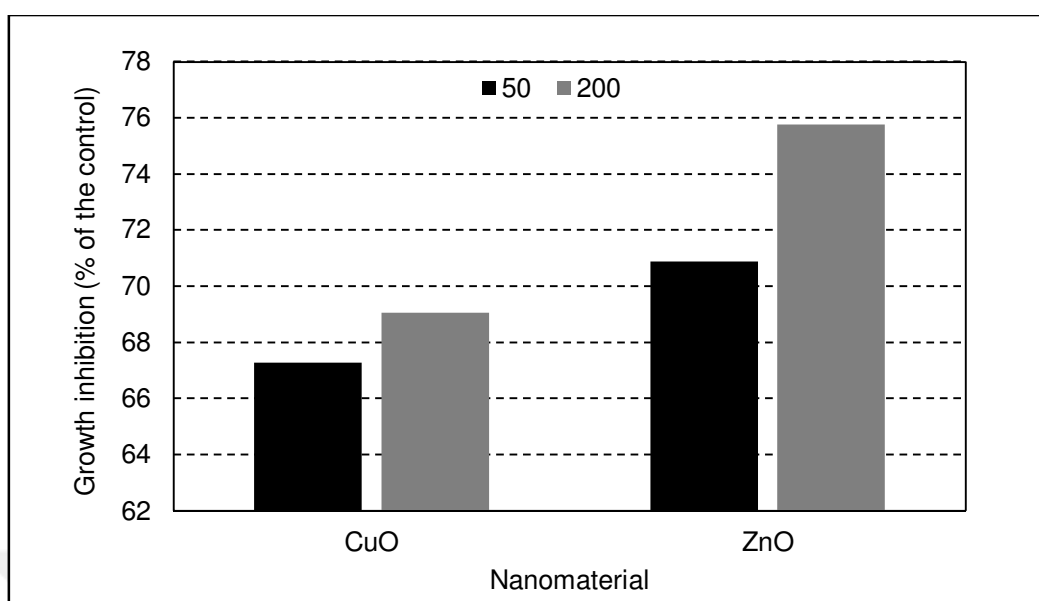


Figure 4.4. Effects of nanomaterial x nanomaterial dose interaction on growth inhibition of gray mold

When growth inhibition of nano CuO and ZnO at 50 and 200 mg/l concentration interaction was in consideration, both nanomaterial had highly significant effect on gray mold growth at 200 mg/l concentration (Figure 4. 4). The inhibitory effect difference of ZnO between 50 and 200 mg/l concentrations was greater than the inhibitory effect difference of CuO doses.

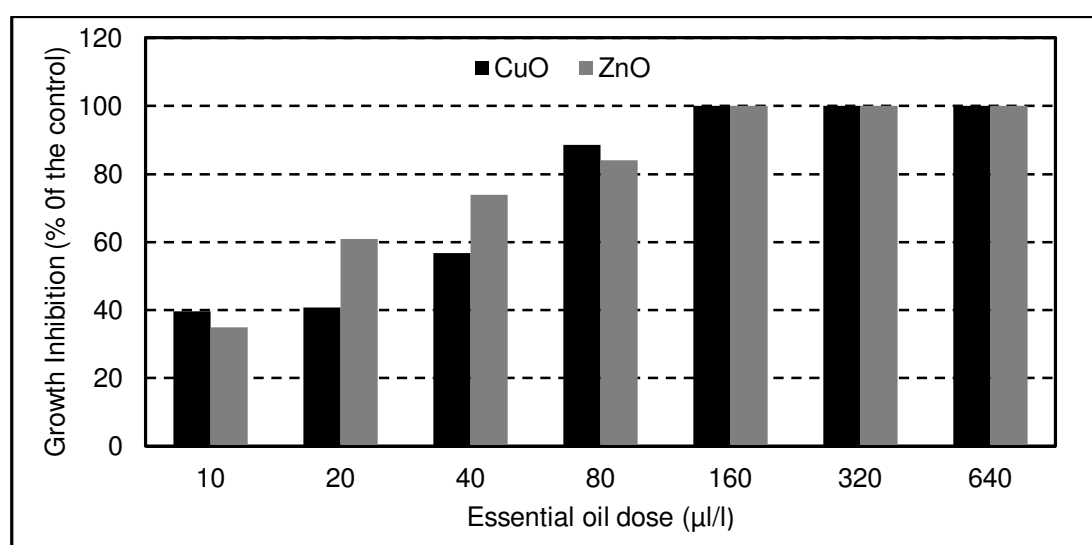


Figure 4.5. Effects of nanomaterial x essential oil dose on growth inhibition of gray mold

In Figure 4.3, showed that nano CuO and ZnO at 160, 320 and 640 $\mu\text{l/l}$ essential oil concentrations had complete inhibition on grey mold growth (Figure 4. 5). However, at 80 μL essential oil concentration CuO had higher inhibitory effect on gray mold growth. But at 0, 20 and 40 μl essential oil concentration nano ZnO had greater inhibition on the growth of gray mold.

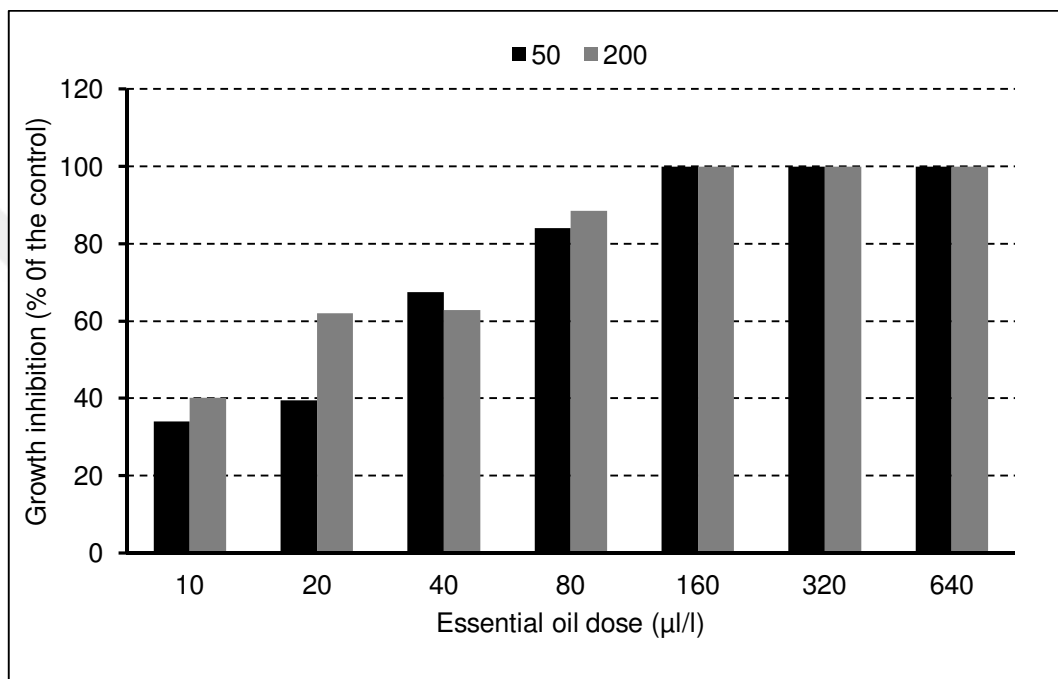


Figure 4.6. Effects of nanomaterial dose x essential oil dose interaction on growth inhibition of gray mold

The biggest differences in the growth inhibition of grey mold were found in the nano material treatments at concentrations of 50 and 50 mg/l with 160 μl essential oil treatments (Figure 4.6.). Complete growth inhibition was achieved at 160 μl essential oil treatments. The 160, 320 and 640 μl essential oil treatments had similar inhibitory effects on gray mold growth either 50 or 200 mg/l nanomaterial concentration.

Table 4.9. Nanomaterial x nanomaterial dose x oregano dose interaction on growth inhibition.

Nanomaterial	Nanomaterial dose(mg/l)	Oregano dose (μl)	Growth inhibition (% percent of the control)
CuO	50	0	18.27
CuO	50	10	36.79
CuO	50	20	25.43
CuO	50	40	57.90
CuO	50	80	100.00
CuO	50	160	100.00
CuO	50	320	100.00
CuO	50	640	100.00
ZnO	50	0	36.30
ZnO	50	10	31.48
ZnO	50	20	53.83
ZnO	50	40	77.28
ZnO	50	80	68.27
ZnO	50	160	100.00
ZnO	50	320	100.00
ZnO	50	640	100.00
CuO	200	0	21.11
CuO	200	10	42.46
CuO	200	20	56.17
CuO	200	40	55.55
CuO	200	80	77.28
CuO	200	160	100.00
CuO	200	320	100.00
CuO	200	640	100.00
ZnO	200	0	29.50
ZnO	200	10	38.39
ZnO	200	20	67.90
ZnO	200	40	70.37
ZnO	200	80	100.00
ZnO	200	160	100.00
ZnO	200	320	100.00
ZnO	200	640	100.00

When nanomaterial x nanomaterial dose, nanomaterial x oregano dose, nanomaterial dose x oregano dose and nanomaterial x nanomaterial dose x oregano dose interactions were considered, it was clearly seen that the significant interaction was resulted from CuO, since complete growth inhibition was obtained at 50 mg/l CuO with 80 μl essential oil concentration while gray mold growth was observed at 200 mg/l CuO with

80 μ l essential oil concentration and essential oil concentration (Table 4.9). Mixing essential oil with nanomaterials enhanced antifungal effect of nano ZnO and CuO.

4.4 Antifungal effect of different concentrations of nanoparticle mixedoregano essentialoil on the whole tomato leaf under *in vivo* conditions.



Figure 4.7. Disease incidence and severity of gray mold.

Dense smooth tan to brown spore bulks on the infected leaf was seen *in vivo* studies (Figure 4.7).

Variance analysis of nanomaterial + oregano essential oil mix on disease incidence of gray mold was given in Table 4.10.

Table 4.10. Variance analysis of nanomaterial + oregano essential oil mix on disease incidence of gray mold.

Variation source	D.F.	Sum of Square	Mean Square	F
Treatment	5	1907.10	1876.63	1579.95**
Error	12	14.25	1.19	
Corrected total o	17	9397.41		
C.V.		4.38		

Variance analysis table showed that nanomaterial + oregano essential treatments were significant in terms of disease incidence (Table 4. 10).

Table 4.11. Disease incidence of gray mold on nanoparticle + essential oil treatment on tomato leaf

Treatment	Incidence (%)
CuO 50 mg/l + 80 μ l/l essential oil	26.16 B
CuO 200 mg/l + 160 μ l/l essential oil	8.26 E
ZnO 50 mg/l + 80 μ l/l essential oil	22.43 C
ZnO 200 mg/l + 160 μ l/l essential oil	7.43 E
Untreated	73.46 A
Fungicide	11.50 D
LSD 0.05	1.93

Variance analysis of nanomaterial + oregano essential oil mix on disease severity of gray mold was given in Table 4.12.

Table 4.12. Variance analysis of nanomaterial + oregano essential oil mix on disease severity of gray mold.

Variation source	D.F.	Sum of Square	Mean Square	F
Treatment	5	294.66	58.93	297.13**
Error	12	2.38	0.20	
Corrected total	17	297.03		
C.V.	6.28			

Variance analysis table showed that nanomaterial + oregano essential treatments were significant in terms of disease severity (Table 4. 12).

Table 4.13. Disease severity of gray mold on nanoparticle + essential oil treatment on tomato leaf

Treatment	Severity (%)
CuO 50 mg/l + 80 μ l/l essential oil	10.40 A
CuO 200 mg/l + 160 μ l/l essential oil	2.46 E
ZnO 50 mg/l + 80 μ l/l essential oil	9.53 C
ZnO 200 mg/l + 160 μ l/l essential oil	1.73 E
Untreated	12.60 B
Fungicide	5.80 D
LSD 0.05	0.79

In this part of the current study various concentration of nanomaterials and essential oil were used *in vivo*. Nanomaterials, CuO (50, 200) mg/l mix with 80 and 160 μ l/l

essential oil and ZnO (50, 200) mg/l mix with 80 and 160 μ l/l essential oil, had great antifungal effect on tomato (Table 4.11 and 4.13). The highest disease incidence was obtained from untreated control plots and the lowest one was obtained from ZnO 200 mg/l + 160 μ l/l essential oil with 7.43%. When disease severity was considered, it varied between 1.73 and 12.60%. The lowest disease severity was obtained from ZnO 200 mg/l + 160 μ l/l essentialoil and the highest was obtained from untreated control treatment.



CHAPTER 5

DISCUSSION

Fungi are responsible for more than 70% of known major crop diseases (Agrios, 2005) and considerable crop losses occur in major World crop species such as rice, wheat, barley, cotton, peanut, and soybean (Dhekney *et al.*, 2007). Gray mold, *Botrytis cinerea*, is one of the economically important pathogens of greenhouse and open-field grown cucumber and tomato. Known chemical controls are not always effective to control gray mold. Therefore, alternative methods in order to protect the crops are very important. Different nanoparticles such as copper, zinc, titanium magnesium, gold, alginate and silver have been developed (Gu *et al.*, 2003, Ahmad *et al.*, 2005, Schabes-Retchkiman *et al.*, 2006). Nanoparticles offer a successful alternative solution for protecting agricultural crops from bacterial and fungal infection.

Essential oils are mostly made up of complex mixtures of monoterpenoids, sesquiterpenes and phenols. The compositions of essential oils vary with the plant species and cultivar of the plant, environmental conditions, harvesting stage, time and processing (Mishra 1994). As displayed by Table 4.1, the essential oils of *O. vulgare*, contained carvacrol, α -myrcene, α terpinene, β - terbipnen, benzene 1-methyl-2-(1-methylethyl), and carvacrol as main components, which showed potent antimicrobial effects. Similar results were reported by Kokkini *et al.*, (1997), Novack *et al.*, (2003), Burt, (2004), Chun *et al.*, (2005) Martino *et al.* (2009) and Jnaid *et al.* (2016) that myrcene, γ -terpinene, α -terpinene, p-cimene, borneol, thymol, carvacrol, β -cariophyllene, limonene, α -pinene, β -pinene, linalool and sabinene were main compounds found in *O. vulgare* essential oils.

It is problematic to connect the activity of a complex mixture to particular components (Pauli and Schilcher 2010). Still, it is reasonable to speculate that the activity of *O. vulgare*, essential oils can be related to the presence of carvacrol, α terpinene, β -

terbipnen and α -thujone. These major essential oil compounds were found to be the most active components (Table 4.1). For most scenarios, the essential oils' prohibitory effects are linked to the dominant elements and not to their other elements (Farag, 1989). Still, some scientists discovered a bigger effect when using the entire essential oil rather than only the dominant compound. This highlights a potential synergistic effect of the minor elements (Bullerman, 1977).

Nanomaterials have the huge potential to protect crop plant by allowing better scientific management of plant diseases as compared to synthetic fungicides and conservation efforts to plant production (Park *et al.*, 2006). Nano zinc oxide and copper oxide are effective antifungal agents. The nanoparticles of Zinc oxide at highest concentration were found the most effective on growth inhibition of gray mold. But inhibition in growth increased with the increase in the concentration of nanomaterials.

The present study revealed the efficacy of different concentrations of CuO and ZnO on the growth inhibition of gray mold. It was clearly seen from the results, that different concentration of CuO and ZnO nanomaterials caused significant inhibition in growth inhibition as compared with the control. The highest inhibition was observed at the highest concentration of nanomaterials followed by lower concentration of nanomaterials, repetitively. The highest growth inhibition of grey mold was observed at the highest nano ZnO concentration followed by lower concentrations of nano ZnO concentrations, respectively. The effect of nano ZnO on the growth inhibition may be due to its antifungal effect on gray mold. Antifungal effect of nano ZnO on some plant pathogenic fungi has also been reported by some workers (Sawai and Yoshikawa, 2004; Violeta *et al.*, 2011; Lipovsky *et al.* 2011; Hassan *et al.*, 2013).

In the present work nano CuO and ZnO were tested for their antifungal activity. Both nanoparticles were found effective on the growth inhibition of gray mold. However, inhibitory effect of ZnO found most effective on the gray mold growth under *in-vitro* condition. Inhibitory effect was found to be dependent on the concentration of nano ZnO as indicated in the current work. The inhibitory effect of nanomaterials could be attributed to the release of extracellular enzymes and metabolites (Perez-de-Luque and Diego, 2009). Antifungal activity of some nanomaterials of ZnO was also reported on some fungi like wood rotting fungi, *Fusarium* species and other phytopathogenic fungi.

It was attributed that antifungal activity of nanoparticles was due to suppression of enzymes and toxins used by the fungal pathogens for pathogenesis (Bhainsa, and D'Souza, 2006; Vahabi *et al.*, 2011).



CONCLUSION

Considering the reduction in mycelial growth and germination of *B. cinerea* *in vitro*, it can be concluded that oregano essential oil could be used as possible bio fungicides, as an alternative to synthetic fungicides, against phytopathogenic fungi on tomato plant, ZnO and CuO nanoparticles possess significant antifungal properties against *B. cinerea* and the inhibitory effects increase as the concentrations of nanoparticles increase. Growth inhibition of nano ZnO on *B. cinerea* were significantly greater than nano CuO. The toxicity of ZnO and CuO on human health and environment must be well studied to offer nano CuO and ZnO as plant protection agents. Since nanoparticles may have greater toxicity than micro ones and both nano ZnO and CuO may induce different levels of cytotoxicity and DNA damage (Karlsson *et al.*, 2009).

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